COMPARATIVE ANALYSIS OF RHD3 MOBILITY IN THE CORITCAL AND NONCORTICAL ER: ASSESSING POSSIBLE INTERACTIONS OF RHD3 WITH THE PLASMA MEMBRANE

An Undergraduate Research Scholars Thesis

by

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Submitted to Honors and Undergraduate Research
Texas A&M University
in partial fulfillment of the requirements for the designation as

UNDERGRADUATE RESEARCH SCHOLAR

Approved by
Research Advisor: Dr. Lawrence Griffing

May 2013

Major: Microbiology
Biochemistry

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ABSTRACT

Comparative Analysis of RHD3 Mobility in Cortical and Noncortical ER: Assessing Possible Interactions of RHD3 with the Plasma Membrane. (May 2013)

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RHD3, an integral membrane protein with active GTPase activity found in plants, has been recently highlighted as an ER remodeling protein. While the function of this protein is unknown, it is proposed that RHD3 acts in the movement of ER tubules to and from the cell cortex via interactions with the cell membrane. Preliminary research has indicated that the GTP-GDP isoform of RHD3 present may act to regulate an interaction with the cell membrane as is observed for small lipid linked G proteins. Another family of proteins known to interact with RHD3 is the reticulons, proteins thought to affect ER tubule stability. By functioning as an anchor for the ER and by interacting with other ER remodeling proteins such as reticulons, RHD3 may change the mobility of other ER remodeling proteins and thus alter ER structural stability. This paper provides an analysis of RHD3 interaction with the cell membrane.

ACKNOWLEDGEMENTS

I would like to thank Dr. Lawrence Griffing for his time spent mentoring me and his help in the process of this research project. Additionally, I would like to extend my gratitude to Dr. Federica Brandizzi who provided the YFP-RHD3 constructs used in this research.

CHAPTER I

INTRODUCTION

The endoplasmic reticulum (ER) is a dynamic membrane system of tubules and cisternae stretching throughout the cell. The mechanisms of ER remodeling however are not well understood. While remodeling of the ER has been shown to be actin dependent, ER structure is not perturbed in the absence of microtubules and actin (Sparkes et al., 2009). Therefore, the ER has a mechanism of stabilizing its structure independent of cytoskeletal elements.

Shaping the ER is a complex process. There are two sets of proteins which appear crucial to proper shaping of the ER, a class of integral membrane proteins called reticulons which are thought to function in the formation of tubules from flattened saccules called cisternae (Sparkes et al., 2010), and another class of membrane proteins that are large GTP-binding proteins related to dynamin, a protein known to wrap around tubular membranes during endocytosis. Furthermore, it appears that these two families interact to form homo- and hetero-oligomeric complexes (Hu et al., 2009; Shibata et al., 2008; Sparkes et al., 2010). The focus of this research is the dynamin-related ER protein in plants, Root Hair Deformed 3 (RHD3).

It has been hypothesized that the dynamin-like GTPases mediate ER-ER tubule fusion events. However, research in plants has shown that, at least in the cortical ER, i.e., the polygonal ER network subjacent to the plasma membrane, tubule fusion is not perturbed in RHD3 deficient cells. Another hypothesis is that this class of proteins mediates the movement of both themselves

and other associated ER proteins, thereby altering the dynamics of other ER remodeling proteins and tubule persistence.

Currently, the movement of ER membrane proteins is using Fluorescence Recovery After Photobleaching (FRAP), a technique that allows the measurement of both the rate of movement and mobile fraction of proteins in the membrane (Sparkes et al., 2009). Preliminary experiments in our lab and published experiments (Stefano et al., 2010) show that cells overexpressing a GDP-locked form of RHD3 contain a set of perturbed endoplasmic (as opposed to cortical) tubules which show dynamics typical of ER membrane proteins. The endoplasmic ER consists primarily of transvacuolar strands which are bundles of tubules associated with actin that traverse the cell. Meanwhile, GTP-bound RHD3 is present in ER showing a normal cortical structure. Therefore, it is hypothesized that the localization of RHD3 within the ER is controlled by GTP mediated interactions with the cell membrane as is observed with many small lipid-linked G proteins in their GTP bound form (Eisenberg et al., 2008; Goryachev et al. 2008).

Due to the cycling between GTP and GDP bound forms of RHD3 and the effects on localization of the ER, RHD3 could actually drive associated proteins and ER movement to and from the plasma membrane. Evidence of such interaction is observed during infection with *Agrobacterium tumefaciens* during which reticulons interact with the bacterial type IV secretion system machinery, implying reticulon association with the plasma membrane (Hwang et al., 2004). This functional association of RHD3 with the plasma membrane will significantly decrease its mobility and that of any associated proteins. An example of this is the integral membrane protein

IST2 which interacts with the plasma membrane. Preliminary experiments in the Griffing and Ryan labs have shown that IST2 has significantly decreased mobility in the cortical ER.

In this study, we were able to transiently express fluorescent fusion proteins of wild type RHD3 and RHD3 that had single amino acid substitutions in regions two different GTP-binding regions of the proteins. The S51N (substitution of arginine for serine at position 51)-RHD3 fusion protein is putatively the GDP-locked form of RHD3. The T75A (substitution of alanine for threonine at position 75) is putatively the GTP-locked form. By examining the movement dynamics of these forms in different regions of the ER, i.e., those associated with the plasma membrane and those not associated with the plasma membrane, a model emerges supporting the hypothesis of the cycling of GTP and GDP bound forms of RHD3 may contribute to membrane dynamics in the ER.

CHAPTER II

MATERIALS AND METHODS

Materials

The YFP-RHD3 constructs used in this experiment were the generous gift of Dr. Federica Brandizzi. The Olympus FV1000 confocal microscope used is part of the Texas A&M University Imaging Center. All other materials were of analytical grade or higher.

Growth conditions

The wild type tobacco was grown in a 1:1 compost-vermiculite mixture with 16 h of light and 8 h dark. All of the plants were grown at 25 C.

Transient expression of constructs in tobacco

Transient expression of the fluorescent constructs in tobacco was induced following the procedure described previously by Sparkes, et al (Sparkes et al. 2006). *Agrobacterium tumefaciens* GV3101::mp90 was transformed with binary plasmids containing the constructs. The *A. tumefaciens* was grown in Luria Broth (LB). Following two washes of the bacteria in infiltration medium, the bacteria were injected at an OD_{600} of .1 to .12 into the leaves of 5-6 week old tobacco plants. Four days after the injection, the injected sites of the tobacco leaves were cut from the plant and viewed using confocal microscopy.

Confocal microscopy

An Olympus FV1000 confocal microscope was used for all imaging performed. A 488 nm Argon laser was used for YFP-RHD3 excitation. For FRAP, a 405 nm high intensity SIM scanner laser was used. The region of interest selected for photobleaching was a 3 µm by 3 µm square. Time lapse images were taken beginning 3 seconds prior to photobleaching. The photobleaching conditions were 100% of the SIM scanner laser power for 3 seconds. The fluorescence recovery was measured for 6 seconds following photobleaching. The time lapse image sets obtained from this process were identically processed in ImageJ by first subtracting 150 from the intensity values of all pixels followed by the use of a median filter with a radius of 1. The latest version of the ImageJ plugin "FRAP_profiler" was used for recovery and percent mobility analysis.

CHAPTER III

RESULTS

Wild type YFP-RHD3

Notably, the mobility of YFP-RHD3 was drastically reduced in the cortical ER versus the transvacuolar strands. Mobility in the so called "ER bundles" was in between these two extremes (Figure 2). Additionally, the half time for recovery was reduced significantly in the cortical ER while almost identical in the transvacuolar strands and ER bundles (Figure 1).

S51N YFP-RHD3

Observing Figures 1 and 2, S51N YFP-RHD3 construct showed its highest mobility of 84% in transvacuolar strands and a similar mobility in the cortical ER. However, a significant reduction of mobility in ER bundles was observed. Compared to the wild-type YFP-RHD3, a relatively small decrease in mobility was observed in the transvacuolar strands while an approximately 3-fold increase in mobility was observed in the cortical ER. Interestingly, a 2-fold decrease in mobility was observed in ER bundles versus the WT construct. The recovery of this construct was somewhat slower than the WT construct with the cortical ER exhibiting the largest difference of a 2-fold increase in the half time of recovery (Figure 2).

T75A YFP-RHD3

Notably, the mobility and recovery of this construct in the cortical ER were very similar to the WT construct. While the mobility remained the same, a greater than 2-fold increase in $t_{1/2}$ was

observed in the transvacuolar strands (Figure 2). Interestingly, the mobility and $t_{1/2}$ in the ER bundles dropped to the level observed in the cortical ER (Figure 1).

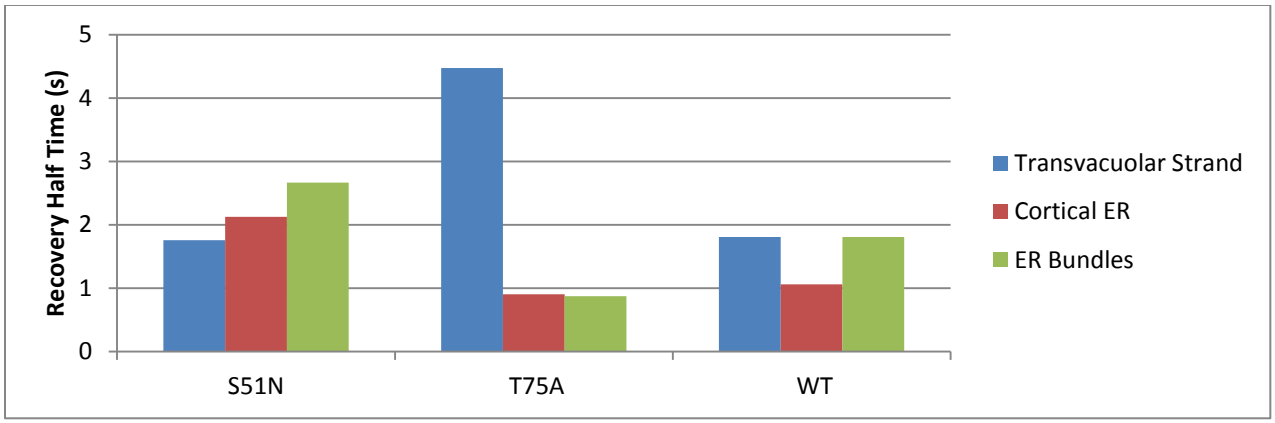

Figure 1. Wild Type and RHD3 Mutants Show Different Patterns of Recovery in the ER. The $t_{1/2}$ values for the WT along with the GTP and GDP bound mutants show different patterns of mobility within the ER, suggesting different activities in the ER. Of special note it the 2-fold increase in recovery time observed between the T75A mutant construct and the WT construct.

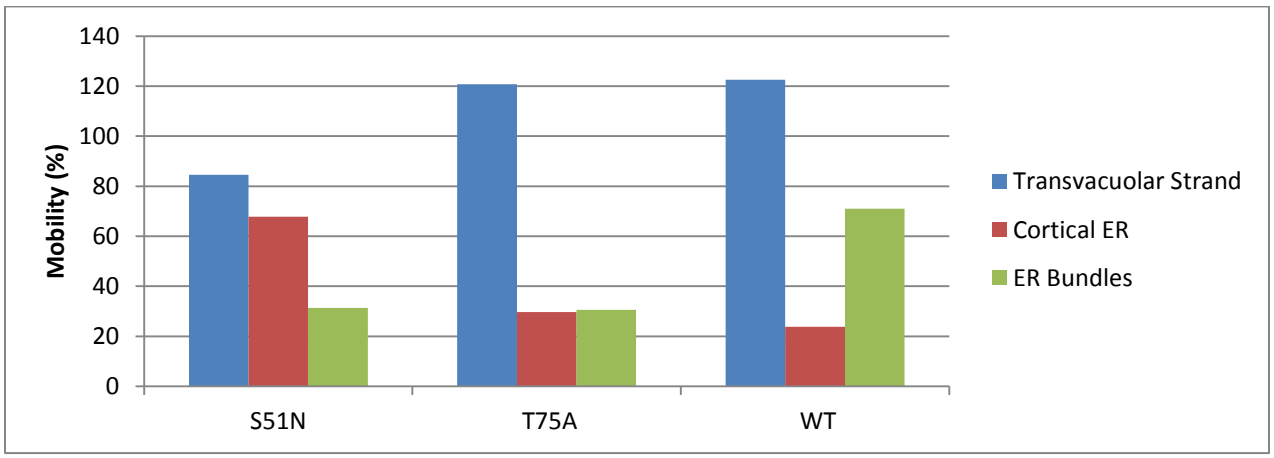

Figure 2. The Wild Type and RHD3 Mutants Show Different Patterns of Mobility in the ER. The percent mobility of the constructs in both different sections and between constructs is different, suggesting different activities of the RHD3 isoforms in the ER. The significant decrease in mobility in the cortical ER compared to the transvacuolar strands in WT YFP-RHD3 suggests that this protein binds to the cell membrane.

CHAPTER IV

CONCLUSIONS

Interaction with the cell membrane

Binding of a protein in the cortical ER to a constituent of the cell membrane would cause the protein to become relatively immobilized, anchored in place due to its connection to both the cell membrane and the ER. Therefore, there should be a significant reduction in the mobility of this protein in the cortical ER as compared to the endoplasmic ER. Analysis of the mobility of WT YFP-RHD3 in the cortical versus endoplasmic ER clearly shows this phenomenon. The observed mobility in transvacuolar strands was 100% mobility while in the cortical ER, a mobility of 23.8% was observed. Therefore, an approximately five fold decrease in mobility was observed in the cortical ER. This suggests a strong interaction with some unidentified constituent of the cell membrane. Therefore, binding of RHD3 to the cell membrane may be the cause of the positioning of the cortical ER just under the cell membrane. Additionally, this same anchoring may be the cause of the relative stability observed in the cortical ER network. By recruiting reticulons and anchoring them due to the interaction with RHD3, the tubular network could be stabilized.

The “ER bundles” identified in this paper were distinguished from transvacuolar strands as they are present near, if not within, the cortical ER as is easily seen with confocal microscopy. Therefore, it has been hypothesized that they are separate from transvacuolar strands found deeper within the cell and that they are an intermediate between these strands and the cortical ER. This would explain the fact that YFP-RHD3 mobility in the ER bundles was different than

the transvacuolar strands identified. ER bundles had a mobility of 70.2%, intermediate between the cortical ER and transvacuolar strands.

Different functions for the GTP and GDP bound isoforms of RHD3

The different constructs showed quite different $t_{1/2}$ and percent mobility values, suggesting that the two different isoforms (GTP and GDP bound) of RHD3 have different functions in the ER. The T75A YFP-RHD3 construct showed a very similar mobility and $t_{1/2}$ in the cortical ER while in the ER bundles and transvacuolar strands the dynamics of this protein were different from the WT construct. It was therefore hypothesized that this isoform is present primarily in the cortical ER. The S51N YFP-RHD3 construct showed increased mobility in the cortical ER, consistent with the hypothesis that this isoform does not bind to the cell membrane. Additionally, a significant approximately 2-fold increase in $t_{1/2}$ is observed between the WT and T75A mutant in transvacuolar strands, indicative of the formation of a homomultimeric or heteromultimeric complex resulting in the decreased motion and recovery observed. A model was proposed based on these findings (Figure 3). The RHD3 isoform represented by the T75A mutant binds the cell membrane and interacts with reticulons to both anchor and stabilize the ER network in the cell cortex. This explains the reduced mobility observed in the cortical ER compared to the transvacuolar strands. The isoform represented by the S51N mutant is capable of homodimerizing on a tubule with another monomer of the same conformation present on a separate tubule. Therefore, the tubules are linked together or bundled as is seen in both ER bundles and transvacuolar strands. By this model, the other isoform of RHD3 regulates and stabilizes the tubule bundles of both transvacuolar strands and, perhaps to a lesser extent based on their proposed status, ER bundles. The S51N mutant shows almost identical recovery in

transvacuolar strands as the WT construct does, suggesting that this isoform of RHD3 is in fact present primarily in the transvacuolar strands.

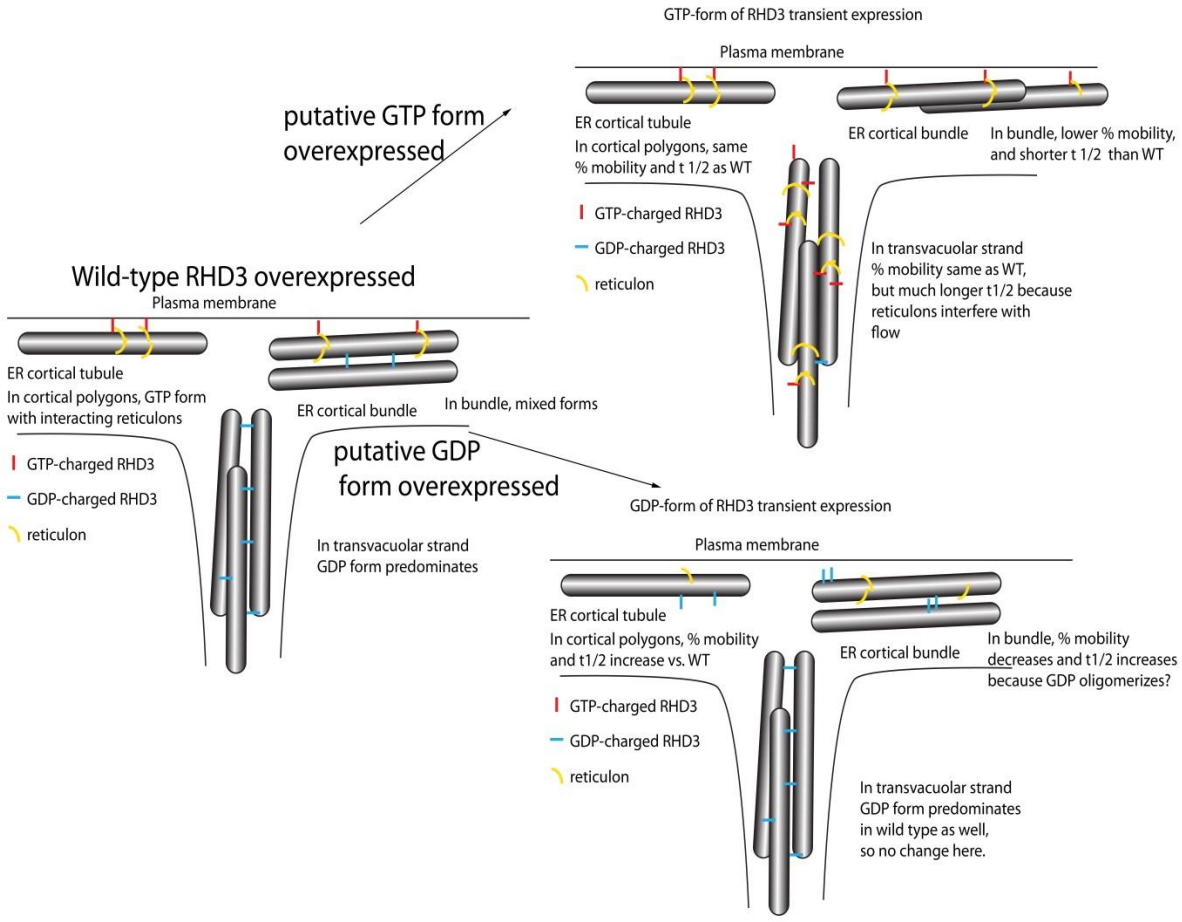

Figure 3. The proposed model for RHD3 function. The GTP and GDP bound isoforms of RHD3 are proposed to have different functions. The RHD3 isoform represented by T75A YFP-RHD3 binds to the cell membrane in the cortical ER, positioning this region of the ER at the cell cortex. Additionally, it interacts with and coordinates reticulons to help induce polymerization. The RHD3 isoform represented by S51N YFP-RHD3 predominates in the endoplasmic ER (transvacuolar strands) where it homodimerizes with a monomer of the same isoform present on another ER tubule, thus causing tubule bundling.

However, a decrease in mobility is observed between the mutant and WT construct. This is hypothesized to be an effect of the overexpression, resulting in RHD3 forming large oligomers rather than simply homodimers and thus decreasing the ability of RHD3 to move within the transvacuolar strands.

GTP cycling may target RHD3 to specific regions of the ER

As previously mentioned, S51N YFP-RHD3 shows the same dynamics in transvacuolar strands as WT YFP-RHD3 while T75A YFP-RHD3 shows the same dynamics in the cortical ER as was observed for the WT construct. This suggests that the isoform of RHD3 represented by S51N YFP-RHD3 is localized in endoplasmic ER while the other isoform represented by T75A YFP-RHD3 is localized in the cortical ER. This could be due to one or both of two events. According to the model proposed by this paper, the RHD3 isoforms stabilize different types of ER (cortical or endoplasmic). Therefore, the form of RHD3 present in a certain place in the ER may determine the fate of the local ER morphology. For ER containing one isoform, the ER would become attached and anchored to the plasma membrane, forming cortical ER. For the other isoform, the ER would become bundled, forming the transvacuolar strands of endoplasmic ER. Another hypothesis is that the GTP cycling acts to target RHD3 to certain regions of the ER in addition its effects on RHD3 activity. Therefore, the different isoforms may be targeted to existing ER structures which they then stabilize rather than causing the formation of new structures. There are many examples of membrane targeting based on GTP cycling such as Rab targeting to the cell membrane and Sar1 targeting to COPII vesicles (Eisenberg, et al., 2008; Hughes et al., 2008). Therefore, the second hypothesis appears to be more reasonable at this time.

Involvement in membrane topology changes is a hallmark of dynamin related proteins

RHD3 is proposed to be involved in regulation of membrane topology via the additional of torsional stress to the membrane. Through an association with RTNs and stimulation of oligomerization, RHD3 is involved in the formation and stabilization of ER tubules primarily within the cortical ER. This is not a novel function for membrane associated dynamin related proteins. Dynamin itself is involved in membrane topology changes via torsional stress. Additionally, the dynamin related proteins Dnm1/Drp1 has been shown to be involved in mitochondrial division in animals and yeast respectively (Friedman et al., 2011). Therefore, an involvement of RHD3 in membrane topology changes is not an ungrounded hypothesis.

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